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DNA Repair Mechanisms and Their Biological Roles in the Malaria Parasite *Plasmodium falciparum*

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SUMMARY

Research into the complex genetic underpinnings of the malaria parasite *Plasmodium falciparum* is entering a new era with the arrival of site-specific genome engineering. Previously restricted only to model systems but now expanded to most laboratory organisms, and even to humans for experimental gene therapy studies, this technology allows researchers to rapidly generate previously unattainable genetic modifications. This technological advance is dependent on DNA double-strand break repair (DSBR), specifically homologous recombination in the case of *Plasmodium*. Our understanding of DSBR in malaria parasites, however, is based largely on assumptions and knowledge taken from other model systems, which do not always hold true in *Plasmodium*. Here we describe the causes of double-strand breaks, the mechanisms of DSBR, and the differences between model systems and *P. falciparum*. These mechanisms drive basic parasite functions, such as meiosis, antigen diversification, and copy number variation, and allow the parasite to continually evolve in the contexts of host immune pressure and drug selection. Finally, we discuss the new technologies that leverage DSBR mechanisms to accelerate genetic investigations into this global infectious pathogen.

INTRODUCTION

DNA double-strand breaks (DSBs) are one of the most deleterious forms of damage that a cell can encounter. A single DSB can potentially lead to loss of heterozygosity (in diploids), chromosome translocations, chromosome loss, cell cycle stalling, overall genome instability, and, ultimately, cell death (1, 2). The repair of DNA damage is closely tied with DNA replication to ensure accurate copying of the genome (3). The DNA repair machinery requires a significant amount of energy to function, a sign of its importance to cell viability. DNA double-strand break repair (DSBR) has been studied extensively over the past 30 years, primarily in the budding yeast *Saccharomyces cerevisiae* (4, 5) as well as in humans (2, 6), in particular

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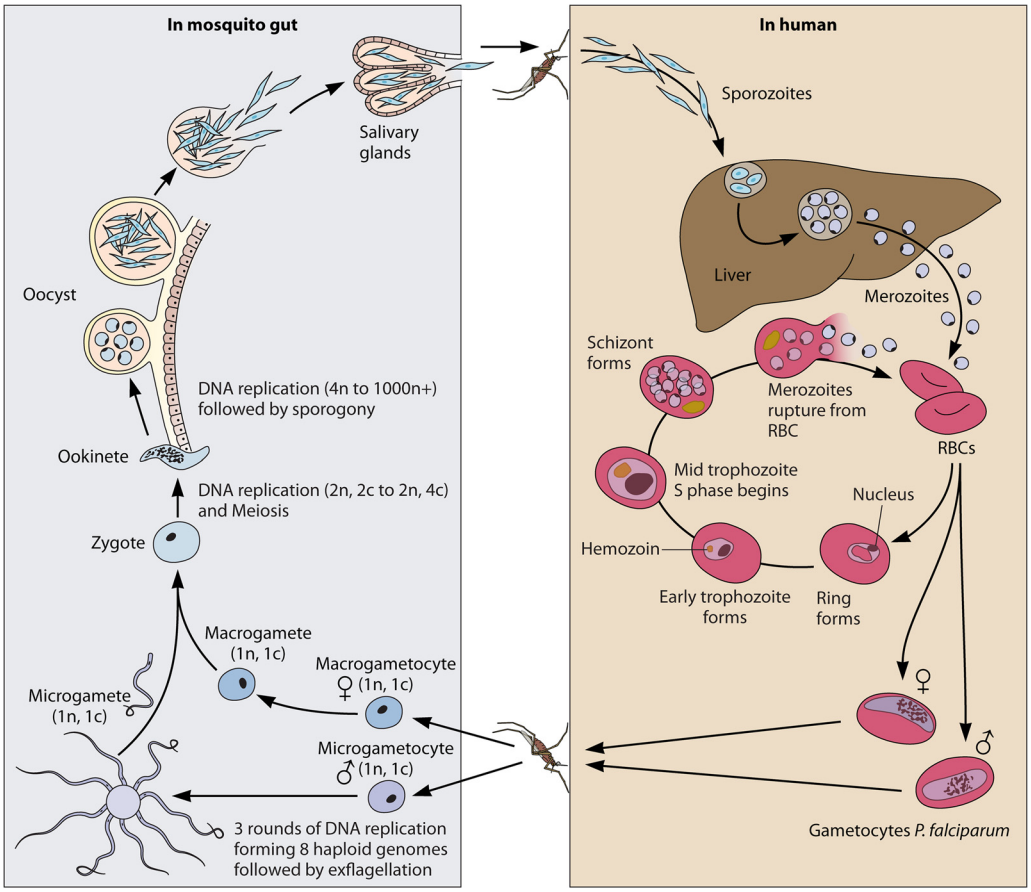

FIG 1 The *Plasmodium* life cycle. A malaria infection begins with the transmission of a *Plasmodium* parasite via a female *Anopheles* mosquito host (left) to a human host (right). After the initial liver stage, the parasite begins its asexual intraerythrocytic cycle. Sexual forms, which develop from the intraerythrocytic parasites, can be transmitted to another mosquito. In the mosquito, parasites undergo meiotic and mitotic replication to form sporozoites, which can infect another human host. RBCs, red blood cells.

because maintaining genome stability is important for preventing cancer.

Despite the destructive potential of DSBs, their role in the cell can also be beneficial. The fidelity of DSBR must be lax enough to allow for sufficient genetic variation. DSBs are created in certain cell types to initiate programmed genome rearrangements, such as meiotic crossing over (5, 7), immune response diversification (8), the mating-type switch of budding yeast (5), and antigenic variation (e.g., in *Trypanosoma brucei* [9]). Genetic manipulation relies on the generation of DSBs by use of site-specific endonucleases. Broken DNA ends are highly recombinogenic (10), and the generation of DSBs is a major rate-limiting factor in recombination (11, 12). Current technologies and strategies rely on the opportunistic use of the DSBR pathways for accurate, efficient, and predictable gene editing (13, 14). Here we first provide a brief overview of DSBR, including key findings established in yeast, and describe the current research on *Plasmodium* orthologs, functions, and unique attributes. We then delve into the use of DSBR in basic parasite processes and the leveraging of DSBR to genetically modify *Plasmodium falciparum*.

THE *PLASMODIUM* LIFE CYCLE

An infected female *Anopheles* mosquito taking a blood meal will secrete its saliva along with *Plasmodium* sporozoites into the dermis of its new host (15) (Fig. 1). Within an hour, these sporozoites migrate to the host's liver and invade hepatocytes. The sporozoites then replicate, forming up to tens of thousands of merozoites, which burst from the hepatocytes to enter the peripheral circulation. Merozoites quickly invade erythrocytes, starting the asexual cycle (16) (Fig. 2). Over the next 48 h, an intraerythrocytic *P. falciparum* parasite produces as many as 24 daughter merozoites, which burst from the host cell to reinitiate a new round of asexual replication. Intraerythrocytic parasites can also adopt an alternative, sexual-stage developmental pathway, in which they form male or female gametocytes. Over a 2-week period, these stages mature and become infectious for mosquitoes (17). Upon transmission, gametocytes convert into gametes that can then mate to form a zygote (15). Following meiosis, zygotes convert into motile ookinetes that exit the blood meal confines and traverse the midgut epithelium, after which they lodge under the basal lamina and form oocysts. During the process of sporogony inside an oocyst, the parasite undergoes multiple rounds of replication to generate thousands of haploid sporozoites, which migrate to the mosquito salivary glands to await the next blood meal.

OVERVIEW OF DNA DOUBLE-STRAND BREAK REPAIR

Causes of Double-Strand Breaks

DSBs can be generated experimentally; others are inherent to cellular processes (5). DSBs are used in many model organisms, and

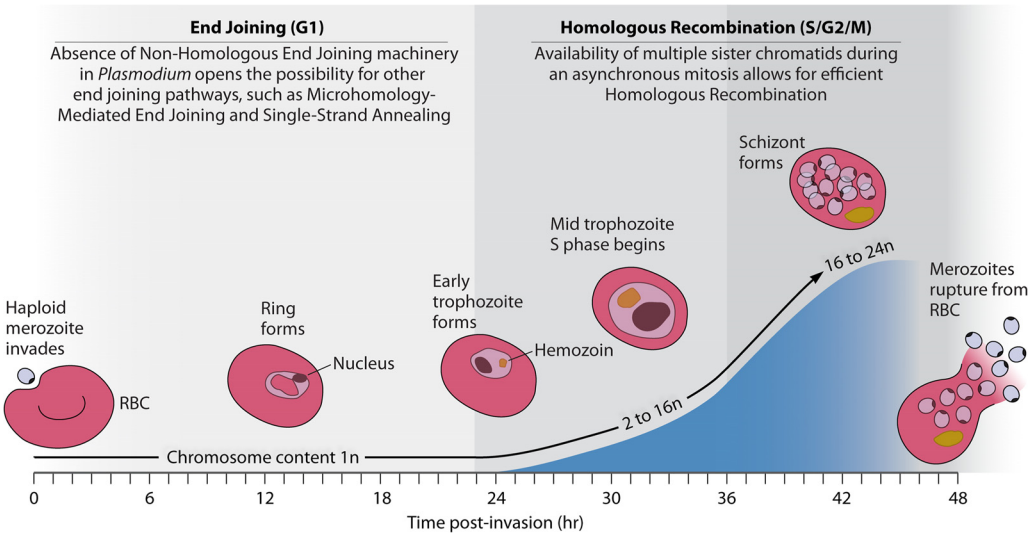

FIG 2 The *Plasmodium* asexual intraerythrocytic cycle. A haploid (1n) merozoite invades a red blood cell (RBC) and develops as the "ring" form from 0 h to about 24 h postinvasion, corresponding to the G_1 phase of the cell cycle. As the parasite transitions from rings to trophozoites, its metabolic activity increases in preparation for DNA replication. Prior to S phase and DNA replication, the parasite is still haploid, allowing for possible alternative EJ pathways. DNA replication produces multiple copies of the genome in an intact nucleus that does not undergo membrane degradation, providing homologous templates for HR. *Plasmodium* DNA replication is asynchronous and can produce a range of sister chromatids, up to about 24n. Nearing the end of the 48-h cycle, each genome is packaged into separate daughter merozoites, which then egress and invade another RBC.

experimental sources include ionizing radiation (IR) (e.g., γ- and X-rays) (18), UV irradiation, chemical mutagens (e.g., hydroxyurea, camptothecin [CPT], and methyl methanesulfonate), and DNA nucleases (e.g., zinc finger nucleases, Tal effector-like nucleases, and the clustered regularly interspaced short palindromic repeats-Cas [CRISPR/Cas] system). Inherent sources can stem from cellular processes, such as the generation of reactive oxygen species by aerobic metabolism (19), transcription (20), and replication fork collapse (21).

Generating double-strand breaks in *Plasmodium*. In comparison to DNA repair research done in model organisms such as yeast, the use of DSB sources to interrogate *Plasmodium* biology has been limited. For example, in *Plasmodium*, irradiation has been used primarily in efforts to create attenuated sporozoite vaccines (22–24), such that parasites are compromised in their intracellular replicative ability. Some chemical mutagens have been used to study nucleotide or base excision repair (NER or BER, respectively) (25–27). CPT, which generates DSBs by trapping the topoisomerase I (TopI) reaction intermediate during relaxation of supercoiled DNA and blocks the progression of the replisome, has been shown to inhibit *P. falciparum* TOP1 (PlasmoDB gene identifier PF3D7_0510500) function *in vitro* (28) and is active against parasite cultures, with a half-maximal inhibitory concentration (IC_{50}) of ~1 μM in a 72-h drug assay (our unpublished results).

Several studies using the first-line antimalarial drug artesunate as a chemical mutagen have emerged in the past decade. The effects of artesunate on mammalian cell lines show that it can promote oxidative DNA damage (29) and also induce a DSBR response (29, 30). However, the far more potent activity of artesunate against *Plasmodium* asexual blood-stage parasites (in the low nanomolar range *in vitro*) and the recent identification of a variant kelch protein (PF3D7_1343700) as a candidate molecular marker of artemisinin resistance (31) suggest a primary mode of antimalarial action distinct from DNA damage.

DNA nucleases, specifically engineered endonucleases, are becoming more commonplace as tools for genetic modification in many organisms, due to their ability to bind and generate DSBs in most investigator-defined DNA sequences (14). These nucleases and their use in *Plasmodium* are described in further detail below.

DNA damage arising from transcription, replication fork collapse, or by-products of normal cellular processes, such as aerobic respiration or hemoglobin degradation, has been studied even less in *Plasmodium*. Replisome collisions with transcription bubbles or single-stranded DNA (ssDNA) generated by oxidative damage can lead to DSBs (20, 32). As an intraerythrocytic parasite grows, it degrades copious amounts of hemoglobin in the digestive vacuole, releasing heme. Heme is oxidized from ferrous (Fe^{2+}) to ferric (Fe^{3+}) iron, producing hydroxyl radicals, a potent DNA-damaging agent (33). Therefore, it is possible that in *Plasmodium*, cellular processes such as hemoglobin degradation and the release of free radicals, coupled with the many rounds of DNA replication, may result in the production of DSBs that the parasite must repair to maintain viability.

DNA DOUBLE-STRAND BREAK REPAIR

In most eukaryotes, the DSBR response can be split into two main branches: the "error-free" homologous recombination (HR) pathway and the potentially "error-prone" end-joining (EJ) pathways. During HR, a broken DNA duplex utilizes a homologous template (a sister chromatid, a homologous chromosome in diploids, a donor plasmid, or an ectopic donor if the DSB forms within a repeated sequence) for highly accurate repair. The EJ pathways do not use a homologous template and instead ligate broken DNA ends together, resulting in a higher possibility of insertions or deletions (indels). Below, we outline DSBR and describe *P. falciparum* orthologs and experimental evidence where possible. We use *S. cerevisiae* nomenclature in reference to orthologs, omitting *Homo sapiens* nomenclature for clarity unless

TABLE 1 Bioinformatic comparison of genes involved in homologous recombination, nonhomologous end joining, and microhomology-mediated end joining among *S. cerevisiae*, *H. sapiens*, and *P. falciparum*

Repair mechanism	Gene product or PlasmoDB ID		
	S. cerevisiae	*H. sapiens*	*P. falciparum*[d]
Homologous recombination	Mre11	MRE11	PF3D7_0107800
	Rad50	RAD50	PF3D7_0605800
	Xrs2	NBS1	
	Sae2	CtIP	
	Exo1	EXO1	PF3D7_0725000
	Sgs1	BLM	PF3D7_0918600
		WRN	PF3D7_1429900[a]
	Top3	TopoIIIα	PF3D7_1347100
	Rmi1	RMI1	
	Dna2	DNA2	PF3D7_1010200
	Rfa1	RPA1	PF3D7_0409600, PF3D7_0904800
	Rfa2	RPA2	
	Rfa3	RPA3	
	Rad51	RAD51	PF3D7_1107400
	Dmc1	DMC1	PF3D7_0816800
	Rad52	RAD52	
		BRCA2	PF3D7_1328200[b]
	Rad54	RAD54	PF3D7_0803400
	DNA polymerase δ	DNA polymerase δ	PF3D7_1017000
	PCNA	PCNA	PF3D7_1361900 (PCNA 1)
			PF3D7_1226600 (PCNA 2)
	Srs2	RTEL1	PF3D7_0514100[c]
	Rad1	XPF	PF3D7_1368800
	Rad10	ERCC1	PF3D7_0203300
	Mus81	MUS81	PF3D7_1449400
	Mms4	EME1	
	Yen1	GEN1	PF3D7_0206000
	Spo11	SPO11	PF3D7_1217100
Nonhomologous end joining	Ku70	Ku70	
	Ku80	Ku80	
	Dnl4	DNA ligase IV	
		DNA-PKcs	
		Artemis	
	Lif1	XRCC4	
	Nej1	Cernunnos/XLF	
Microhomology-mediated end joining	Mre11	MRE11	PF3D7_0107800
	Rad50	RAD50	PF3D7_0605800
	Xrs2	NBS1	
	Sae2	CtIP	
	Tel1	ATM	
	Rad1	XPF	PF3D7_1368800
	Rad10	ERCC1	PF3D7_0203300
	Rad27	FEN1	PF3D7_0408500
	Cdc9	DNA ligase I	PF3D7_1304100
	Rev3	DNA Pol ζ catalytic subunit	PF3D7_1037000
	Pol4	DNA Pol β	
	Rad30	DNA Pol η	

[a] Lacks 5′-to-3′ exonuclease indicative of WRN.
[b] Low homology.
[c] Possible UvrD helicase.
[d] *P. falciparum* orthologs were determined from previous publications or were confirmed by BLAST and identification of unique domains with Pfam, version 27.0.

otherwise stated. *P. falciparum*, *S. cerevisiae*, and *H. sapiens* orthologs can be found in Table 1. For more in-depth reviews of DSBR, we refer the reader to several excellent publications (1, 2, 4, 5, 34).

Mitotic Recombination: Mechanisms and Evidence in *Plasmodium*

Initial double-strand break sensing and resection. A DSB is first sensed by the Mre11-Rad50-Xrs2 (MRX) complex (Fig. 3A) (35).

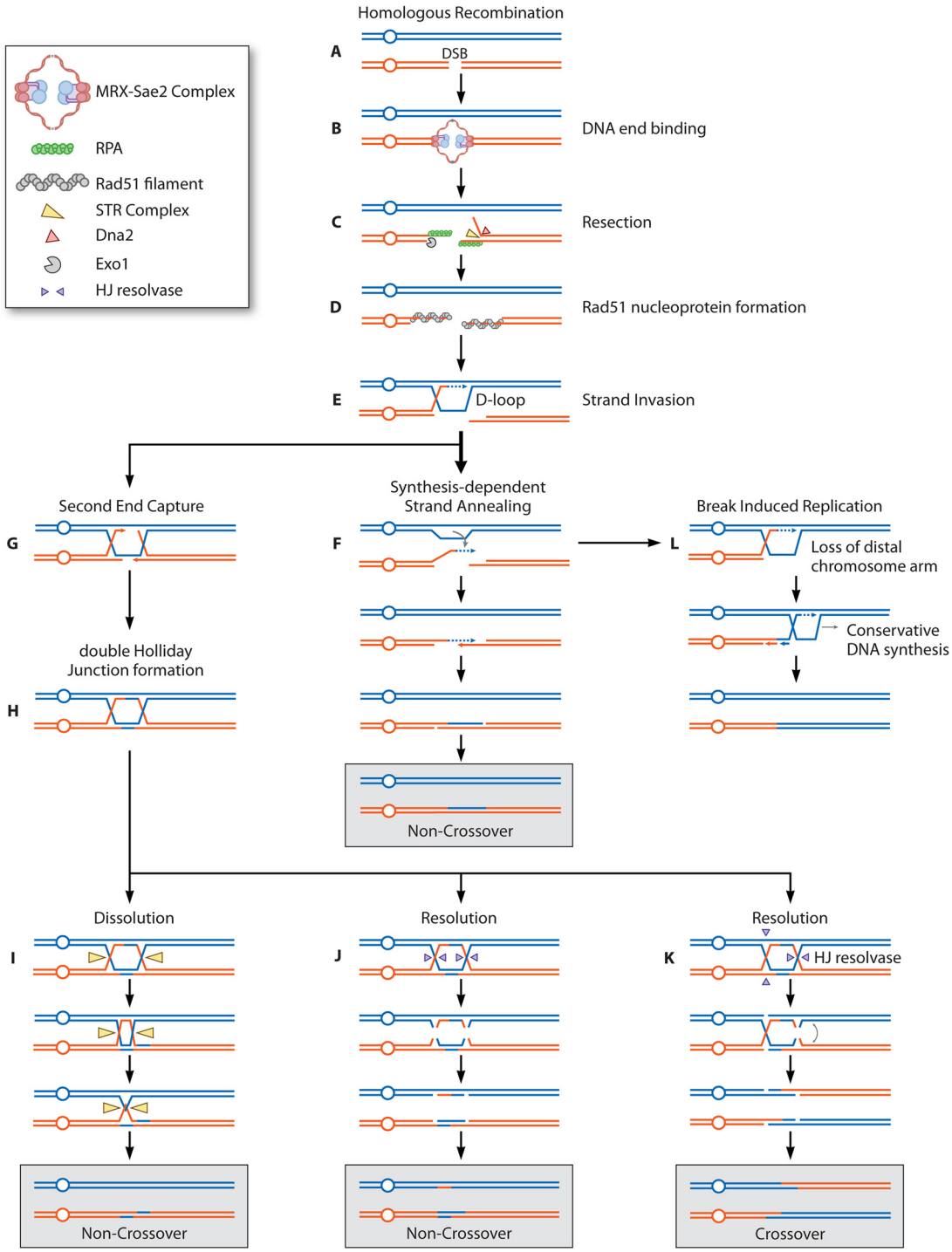

FIG 3 Canonical models of homologous recombination, synthesis-dependent strand annealing, and break-induced replication. A double-strand break (DSB) in a chromosome (A) is first sensed and tethered together by the MRX-Sae2 complex (B). (C) Resection by Exo1 (gray "Pac-man") or the STR-Dna2 complex (yellow and red triangles, respectively) exposes 3′ ssDNA tails, which are then bound by RPA (green dots). RPA is replaced by Rad51 (gray helices) (D), which forms a nucleoprotein filament that invades homologous templates (E). (F) Synthesis-dependent strand annealing (SDSA) uses the invading 3′ tail as a primer for DNA synthesis. Once sufficient homology is synthesized, the invading strand can bridge the DSB to restore chromosome integrity. (L) If the distal chromosome arm is lost, then break-induced replication can occur, with DNA synthesis continuing conservatively until it reaches the end of the chromosome arm. If both broken DNA ends are captured by a homologous template (G), then a double Holliday junction (dHJ) can be formed (H). This structure can be resolved in a number of ways. (I) Merging of both Holliday junctions by the STR complex (yellow triangles) dissolves the dHJ in a hemicatenane structure, which is unlinked by the Top3 topoisomerase. Cleavage of both Holliday junctions by structure-specific resolvases (purple triangles) can unlink the dHJ to produce either noncrossover (J) or crossover (K) products.

The MRX complex binds to double-stranded DNA (dsDNA) ends formed by a DSB, positions both ends in close proximity, and, in collaboration with Sae2, initiates 5′-to-3′ resection, creating short 3′-terminated ssDNA tails (Fig. 3B). Initial resection is a rate-limiting step in HR, as it provides the substrates for further, extensive resection (36, 37). Mre11 and Rad50 show high homology with their *Plasmodium* orthologs, but Xrs2 and Sae2 do not, likely due to low sequence conservation among all species.

Extensive resection. Initial resection is followed by 5′-to-3′ extensive resection by functionally redundant factors (exonuclease 1 [Exo1] and the Sgs1-Top3-Rmi1-Dna2 [STR-Dna2] complex) (36, 37), which commits DSBR to HR and generates long 3′ ssDNA tails (Fig. 3C). Exo1 degrades DNA from the 5′ to the 3′ end and is a member of the 5′-structure-specific Rad2/XPG family of nucleases, which are involved in most DNA repair pathways (e.g., mismatch repair [MMR] and NER). Sgs1 is a RecQ helicase that unwinds linear dsDNA, creating a Y-shaped structure that is then cleaved by the flap endonuclease Dna2 (Fig. 3C) (38). Sgs1 has two human homologs, BLM and WRN (mutated in Bloom's and Werner's syndromes, respectively). BLM has been shown to be the primary resection protein in mammalian cells (39, 40), although *Xenopus* WRN-DNA2 can also resect linear DNA substrates *in vitro* (41). Both BLM and WRN have *P. falciparum* homologs (PF3D7_0918600 and PF3D7_1429900, respectively), though the *P. falciparum* WRN homolog lacks the 5′-to-3′ exonuclease domain that is characteristic of WRN proteins in other eukaryotes. It is therefore possible that PF3D7_1429900 is in fact a different RecQ helicase.

The extent of resection varies between species. Yeast can exhibit ssDNA tract lengths of up to 2 to 4 kb during mitotic HR and up to 850 bases during meiotic HR (34). Detailed analyses of resection have not been performed in *Plasmodium*. Extensive ssDNA resection tracts are likely to signal cell cycle arrest and prevent unwanted recombination by exposing more unique sequence to search for a homologous template (34). In an A/T-rich organism with extensive regions of low complexity, such as *P. falciparum*, long resection tracts may be beneficial for ensuring that a resected sequence is unique enough to undergo HR with the correct template.

Rad51 nucleoprotein filament formation and strand invasion. 3′ ssDNA tails generated by resection are first coated by the heterotrimeric replication protein A (RPA) complex (Fig. 3C), protecting them from degradation and secondary structure formation (42). *P. falciparum* encodes all three subunits of RPA and an additional truncated version of the RPA1 subunit (RPA1S), which antagonizes the long form (RPA1L) during *in vitro* recombination (43). Yeast Rad52 (or the human breast cancer type II susceptibility protein [BRCA2]) then displaces RPA and simultaneously delivers ATP-bound Rad51, the central HR recombinase, onto ssDNA, allowing it to form a nucleoprotein filament (Fig. 3D). Rad51 catalyzes invasion and pairing between the ssDNA to which it is bound and the complementary sequence to form a displacement loop (D loop) (Fig. 3E).

Rad52 homologs have not been found in *Plasmodium* (44), and the *P. falciparum* RPA1 homologs lack similarity to the N-terminal domains of other eukaryotic RPA1 orthologs (45) that are necessary for Rad52 interactions (46). *Caenorhabditis elegans*, *Drosophila melanogaster*, and *Arabidopsis thaliana* all lack Rad52, which may also be the case for *P. falciparum*. Mammals use BRCA2 in addition to Rad52, and BRCA2 sequence similarity even among mammals is relatively low. Nevertheless, BRCA2 homologs have been briefly mentioned for *P. falciparum* (PF3D7_1328200) and *Plasmodium yoelii* (PY17X_1348100), where homology is found in only 6 BRC repeats. These repeats are known to stimulate Rad51-ssDNA binding and to prevent nonspecific Rad51-dsDNA binding (47). *Trypanosoma cruzi*, *T. brucei*, and *Leishmania major* also have BRCA2 homologs with various numbers of BRC repeats (48). Other BRCA2 regions (the oligonucleotide-binding [OB] fold that binds ssDNA and the Tower domain, which binds dsDNA), however, have not been identified in these homologs. These regions also cannot be identified in the *D. melanogaster* BRCA2 homolog (47) and may be too divergent to detect bioinformatically, as they can have low sequence conservation (49). Nevertheless, *D. melanogaster* BRCA2 remains proficient in homologous recombination (50), as is expected to be the case with *Plasmodium* parasites. In stark contrast to Rad52 and BRCA2, *P. falciparum* Rad51 shows high homology to Rad51 proteins of many species. Predictably, it is upregulated in response to the DNA-damaging agent MMS, performs strand exchange on DNA substrates *in vitro*, and hydrolyzes ATP (44, 51).

Efficient Rad51-mediated D-loop formation is enhanced by the Rad55-Rad57 heterodimer and the Rad54 motor protein. Rad55-Rad57 stabilizes the Rad51 nucleoprotein filament by preventing Rad51 displacement from ssDNA (52). Rad54 is a dsDNA-dependent ATPase that translocates along dsDNA, enhances Rad51-dependent strand exchange, and stabilizes Rad51 filament formation on both ssDNA and dsDNA (53–55). Rad54, however, only dissociates Rad51 from dsDNA. The synergistic effects of Rad54 on Rad51-mediated strand exchange are mirrored in *in vitro* assays using purified *P. falciparum* Rad51 (PfRad51) and PfRad54 (PF3D7_0803400) (43).

D-loop formation is followed by polymerase δ-dependent DNA synthesis primed from the 3′ OH group of the invading 3′ ssDNA tail (Fig. 3E). Synthesis extends for ~250 bp in yeast (56), expanding the D loop and incorporating any single nucleotide polymorphisms (SNPs). So far, extension of the invading arm has been found to incorporate SNPs 150 bases distal to the DSB with a high frequency (13), and up to 900 bp distal at a lower but substantial level, during gene editing in *P. falciparum* (57). More detailed frequencies and tract lengths have yet to be delineated thoroughly.

Noncrossovers (NCOs) and crossovers (COs). Two basic options exist after D-loop formation: synthesis-dependent strand annealing (SDSA) (Fig. 3F) and formation of double Holliday junctions (dHJs) (Fig. 3H). During SDSA, the invading strand is extended to traverse the DSB, the D loop is collapsed, and the extended strand bridges the DSB. The homologous template is left unchanged. Possible SNPs copied from the homologous template will generate heteroduplex DNA and be corrected by the MMR machinery. MMR tends to favor the donor as the "correct" template, but the mechanism of this proclivity has yet to be determined (56).

If the D loop captures the second 3′ ssDNA tail (Fig. 3G), then the D-loop intermediate can form a dHJ (Fig. 3H). dHJs are then processed by two distinct, canonical pathways. dHJs can be "dissolved," whereby they are merged together by the Sgs1-Top3-Rmi1 (STR) complex into a hemicatenane structure, which is unlinked by the topoisomerase Top3 to generate an NCO product (Fig. 3I). Alternatively, dHJs can be resolved by Mus81-Mms4, Yen1, or Slx1-Slx4 (58). Depending on the cleavage pattern of the

dHJs, resolution will form NCO or CO products (Fig. 3J and K, respectively). Currently, more intricate pathways branching from the canonical model of dHJ resolution are being elucidated (58, 59). Some of the nucleases, such as Mus81, Yen1, Rad1, and Rad10, have orthologs in *P. falciparum* and are listed in Table 1. Orthologs could not be found for Mms4, Slx1, and Slx4.

By definition, HR by any of these mechanisms is highly accurate, but errors can arise. For example, mitotic crossovers in diploids can lead to loss of heterozygosity. Nonallelic HR (NAHR) between repeated sequences can generate copy number variants (CNVs) (discussed below) or translocations. Furthermore, if a chromosome arm is lost during repair, the invading strand intermediate can copy the homologous template until the site of its telomere, using a process termed break-induced replication (BIR) (Fig. 3L). BIR can cause extensive loss of heterozygosity in diploids (60). In *Plasmodium*, BIR is one mechanism that is believed to generate novel *var* sequences (see below).

Meiotic Recombination: Mechanisms and Evidence in *P. falciparum*

Meiotic recombination is important for the efficient production of sporozoites in *Plasmodium* (61), spores in budding yeast (62, 63), and gametes in mammals (7, 64). The basic mechanistic outcomes of meiotic recombination are largely similar to those of mitotic recombination, but they involve a larger set of meiosis-specific proteins for the programmed generation of genetic diversity (7, 63). Here we briefly discuss meiosis in *Plasmodium* and focus on the meiotic factors involved in recombination.

The mosquito stage of the *Plasmodium* life cycle begins with transmission of male and female gametocytes via a blood meal (Fig. 1). Almost immediately, the male microgametocyte undergoes three rapid rounds of DNA replication, producing eight haploid genomes (65). Exflagellation produces eight single microgametes (1n) (66). A microgamete can subsequently fertilize a female macrogamete (1n), producing a zygote (2n). A subsequent round of DNA replication (2n, 4c) is then followed by meiosis (67, 68) to form a tetraploid ookinete (2n, 4c). During meiosis, crossing over via HR is known to be important for the formation of yeast spores (62) and begins with the production of programmed DSBs by the meiosis-specific topoisomerase-like protein Spo11 (69). Note that *Plasmodium* orthologs of Spo11 exist, suggesting a conserved mechanism (70). In yeast, meiotic HR utilizes the Rad51 homolog Dmc1 to catalyze strand exchange, with Rad51 playing a supporting role (71). In *Plasmodium berghei*, a Dmc1 knockout produces fewer and smaller oocysts and substantially smaller numbers of sporozoites (61), implicating marked defects in meiotic recombination. While a similar knockout has not been studied in *P. falciparum*, Dmc1 is expressed in gametocytes prior to meiotic recombination, implicating a similar role (72).

As with mitotic HR, successful strand invasion (Fig. 3E) provides the choice between forming NCOs and COs by various pathways (Fig. 3F and H). Spo11-induced DSBs are predominately repaired as NCOs (likely by SDSA) and COs at a 10:1 ratio in mammals (7). This relative excess of NCOs has also been seen in *P. falciparum* (73, 74). However, these reports may still underrepresent the number of NCOs, due to a lack of resolution or to NCO products that are indistinguishable from either parental chromosome.

In contrast to the case for mitotic HR, resolution of meiotic dHJ intermediates in *S. cerevisiae* requires the MutL homologs Mlh1 and Mlh3 along with Exo1 (7, 62). In the case of *P. falciparum*, whole-genome sequence analysis of artemisinin-resistant Cambodian parasite populations showed a high frequency of an Mlh1 (PF3D7_1117800) mutation (75), possibly implicating a defect in meiotic crossing over. In a yeast *exo1*Δ strain, the homologous Mlh1 mutation showed a moderate mutator phenotype (76), consistent with a loss of function. To date, no Mlh3 ortholog has been identified in *P. falciparum*. During meiotic recombination, each chromosome averages one crossover (73, 74, 77) and has a genetic map unit distance of ~10 kb (77) to ~15 kb (73) per centimorgan. Meiosis is followed by many rounds of DNA replication inside oocysts to form thousands of genomes that segregate into individual haploid sporozoites (66, 78).

Investigations into the process of meiotic recombination in *Plasmodium* have been limited to analyzing *P. berghei* DNA content during sexual reproduction (65, 67). Several other studies, nonetheless, have explored outcomes of meiotic recombination. *P. falciparum* genetic cross studies have analyzed the segregation of chromosomal markers (SNPs and microsatellites) spread out over the genome between geographically distinct parasite clones: 3D7 × HB3 (79), HB3 × Dd2 (73, 74, 80–82), and 7G8 × GB4 (77, 83, 84). These were instrumental in mapping the dihydrofolate reductase gene (*dhfr*) (85) and *P. falciparum* chloroquine resistance transporter gene (*pfcrt*) (86) that are key drug resistance loci and identifying key determinants of host cell tropism (77, 83, 84).

End Joining

Basic mechanisms of end joining. Parallel to HR, the EJ pathways are comprised of the classical and alternative EJ pathways, by which broken DNA ends are religated without a homologous template for repair. The classical nonhomologous end-joining (NHEJ) pathway (Fig. 4A) has commonly been referred to as the "error-prone" pathway, though it is likely rather error-free but accommodating of promoting genetic variability (87).

In yeast, NHEJ can be performed with just the Ku70/80 (Ku) heterodimer and the DNA ligase IV-Lif1-Nej1 complex (88). The Ku heterodimer binds broken DNA ends, which can protect them from end resection and commitment to HR (89) and act as a platform for NHEJ factor recruitment (88). Ku promotes EJ of a variety of substrates by sterically fitting in the grooves of the DNA double helix, as opposed to forming specific base interactions (90). In vertebrates, the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and the endonuclease Artemis are essential NHEJ factors. DNA-PKcs and Artemis bind Ku-bound ends, and phosphorylation of Artemis by DNA-PKcs activates its endonuclease activity (91). Either nucleolytic degradation or polymerase nucleotide addition generates DNA ends compatible for EJ.

Absence of nonhomologous end joining in *Plasmodium*. To date, bioinformatic analyses have failed to identify any *Plasmodium* homologs of NHEJ proteins (Table 1) (92). In contrast, another apicomplexan parasite, *Toxoplasma gondii*, carries a functional Ku-dependent NHEJ pathway (93), suggesting that despite relative evolutionary proximity to *T. gondii*, *Plasmodium* has lost its NHEJ machinery. In support of this hypothesis, a recent study showed the absence of any NHEJ products recovered from *in vivo* endonuclease-generated DSBs in *Plasmodium* (13). Furthermore, other eukaryotic pathogens, such as *Giardia lamblia* (94), *Encephalitozoon cuniculi* (95), and *Trichomonas vaginalis* (96), also lack NHEJ components.

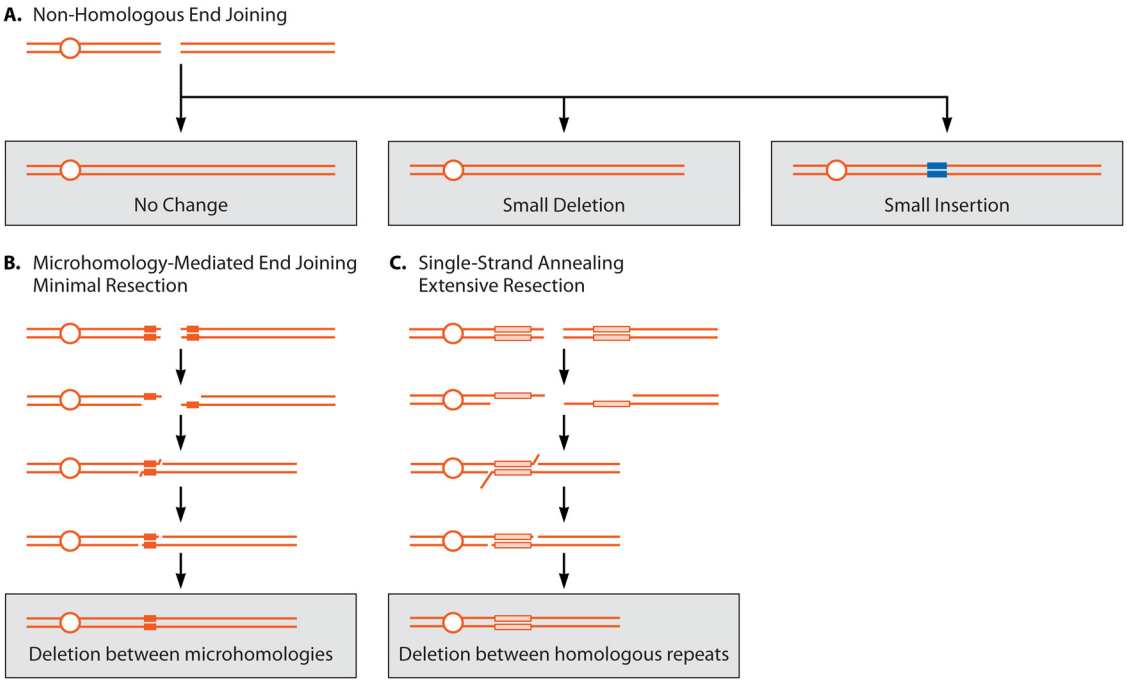

FIG 4 EJ pathways. (A) Nonhomologous end joining (NHEJ) involves the joining of broken DNA ends with little to no homology. A simplified model of NHEJ depicts the restoration of the original DNA sequence and introduction of small insertions (blue strands) or small deletions. (B) Microhomology-mediated end joining (MMEJ) relies on minimal resection by proteins shared with HR to expose short homologies of up to 25 bp, which can anneal, deleting any intervening sequence. (C) Single-strand annealing (SSA) involves longer stretches of resection. Rad52 (not shown) in yeast displaces RPA and anneals longer homologies, generating larger deletions.

Possible alternative end-joining mechanisms in *Plasmodium*. If NHEJ was indeed lost during *Plasmodium* evolution, then alternative EJ pathways may still be active. In yeast, *ku*Δ mutants revealed pathways that occur at lower frequencies than that of NHEJ (97). These Ku-independent processes include microhomology-mediated end joining (MMEJ) (Fig. 4B) and single-strand annealing (SSA) (98) (Fig. 4C). Both pathways require resection to expose homologies internal to the DSB ends. MMEJ refers to joining between microhomologies (up to 25 bp), whereas SSA occurs between more extensive homologies. Yeast Rad52 promotes annealing of the complementary ssDNA during SSA (99), by displacing RPA coating ssDNA (100), but plays little or no role in MMEJ. As discussed above, BRCA2, but not Rad52, is homologous to *P. falciparum* PF3D7_1328200. The *Ustilago maydis* BRCA2 homolog, Brh2, is reported to promote strand annealing similar to the reaction catalyzed by Rad52 (101), suggesting that the *Plasmodium* BRCA2 homolog may fulfill a similar role. Nonetheless, current evidence suggests that SSA is not a major mechanism of DNA repair in *Plasmodium* (13). For another eukaryotic pathogen, *T. brucei*, chromosomal and *in vitro* plasmid EJ assays produce only MMEJ-generated repair products (102–104). NHEJ is not used, and *T. brucei* Ku functions only in telomere maintenance (105).

A recent report (106) suggests that *P. falciparum* may be able to repair a DSB by an alternative EJ mechanism whereby a few bases are added to the broken ends, thereby providing microhomologies. The frequency of these events is very low, and larger deletions (e.g., possible SSA products) beyond the locus analyzed were not examined. Nevertheless, *Plasmodium* species carry all necessary components of the MMEJ machinery, many of which overlap those for HR (Table 1). However, given the general lack of NHEJ, MMEJ, and SSA products observed in *Plasmodium*, it seems unlikely that EJ processes occur to any significant extent in malaria parasites.

Potential implications of the absence of end joining in *Plasmodium*. The absence, or at least highly infrequent use, of EJ pathways in *P. falciparum* may be an important factor in the production of a live, radiation-attenuated sporozoite vaccine. Haploid sporozoites in the Sanaria PfSPZ vaccine are metabolically active yet nonreplicating (23, 24, 107). PfSPZ is generated with sporozoites dissected from infected mosquitoes exposed to 15 krad of γ-irradiation. Sufficient irradiation introduces DNA damage (108) without compromising hepatocyte invasion, gene expression, and initial trophozoite development, but it prevents nuclear division (109). Estimations from yeast data suggest that the 15-krad dose is sufficient to generate small but sufficient numbers of DSBs (110). Therefore, the crucial replication defect of irradiated sporozoites may result from the parasite's lack of an efficient EJ pathway during this strictly haploid stage and may manifest itself only when DNA replication occurs during liver-stage proliferation.

Studies of irradiated blood-stage *P. falciparum* support this notion. Experimental analyses showed that cellular distress is dose (111) and cell cycle (112) dependent. Studies with parasites subjected to various IR doses throughout the asexual blood stage (Fig. 2) showed that ring-stage parasites and multinuclear schizonts with a 1c chromosome content cannot reconstitute an *in vitro* culture after IR exposure as efficiently as the case with trophozoites, which have a chromosome content of >2c (112, 113). Given that the trophozoite stage has numerous sister chromatids as templates for repair in a syncytium, the IR-induced DNA damage is

likely readily repaired by HR. Further studies are required to discern whether the segregation of chromosomes after DNA replication (78) inhibits efficient HR. Altogether, without a robust EJ pathway, the data show that it is possible that *Plasmodium* is more sensitive to DNA damage than other model organisms, such as yeast and humans.

HOMOLOGOUS RECOMBINATION IN *P. FALCIPARUM*

Barring a few exceptions, DSBR in *Plasmodium* is rarely studied solely at a mechanistic level. Most published studies examine the products of such processes in the context of drug resistance, antigenic diversification, population structure, or genetic manipulation. Together, these studies each contribute to a larger picture of the consequences of *Plasmodium* DSBR, but few provide direct experimental evidence of its underlying molecular intricacies. Nevertheless, the compiled picture shows a unique organism in which DSBR dictates a broad spectrum of phenotypes.

Subtelomeric Regions and Antigenic Diversification

Unlike the case in *T. brucei*, antigenic variation in the *Plasmodium* parasite does not occur by HR. Whereas *T. brucei* undergoes Rad51-dependent gene conversion to replace the active antigen-encoding gene (the variant surface glycoprotein gene [*VSG*]) with one of the many inactive pseudogenes (114) in order to evade host defenses, *P. falciparum* antigenic variation is mediated at the epigenetic level (115). *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which is presented on the host's infected erythrocyte and exposed to the immune system, is encoded by the clonally variant *var* gene family. *var* does not require a DSB to switch between its family members and instead epigenetically silences all but one *var* cassette among the ~60 total per parasite (115).

Though recombination plays no role in antigenic variation, it does mediate *var* gene antigen sequence diversification and *var* gene family composition during mitotic (116–118) as well as meiotic (77, 119) cell cycles. *var* genes are located primarily in subtelomeric regions of chromosomes (120, 121), although some are also found clustered in central chromosomal regions (92, 121, 122). The subtelomeric regions are also home to the *rifin* and *stevor* multicopy gene families, which also interact with host factors. Spatial positioning studies show that chromosome ends are clustered together at the nuclear periphery (121–123). In yeast, spatial proximity of a DSB to a donor template greatly enhances HR (124). Therefore, in addition to providing a mechanism for selective *var* expression, clustering of subtelomeric ends may provide the close proximity between heterologous chromosomes for recombination to generate novel *var* sequences (122).

The rate of recombination in these subtelomeric regions has been shown to be much higher than in the core chromosome: for *in vitro* 3D7 cultures, the mitotic recombination rate is about 4.7×10^{-6} events per base pair per generation, with over 80% of events occurring in the subtelomere (117). Parasite populations from different global regions reaffirm this notion (119). It is possible, nevertheless, that both mitotic and meiotic recombination contribute to recombination in subtelomeric regions, as both can occur during the mosquito stages. This may provide parasites with the ability to persist longer in a single blood-stage infection by forming novel *var* sequences and, thus, distinct PfEMP1 antigens (116).

Mechanistically, the diversification of *var* sequences and *var* gene family composition can be driven by a number of recombination pathways. Reports pinpointing the precise mechanisms driving these events have not been published, although insights can be gleaned by analyzing the recombination products. For example, a *var* allele can copy a portion of a donor allele to produce a novel, chimeric allele. In mitotically dividing *P. falciparum* cells, studies suggest that this process is mediated by an SDSA-mediated gene conversion event (116, 117) (Fig. 3F) and by BIR (Fig. 3L) (117). Intragenic COs between two *rif* genes can create two novel sequences (116). NAHR (discussed below) (Fig. 5A) may also delete or amplify large swaths of sequence in subtelomeric regions, thereby altering *var* gene family composition for a given parasite (117, 118).

For the sexual stages, a recent study has shown that some hot spots for ectopic *var* recombination are energetically likely to form DNA secondary structures, which may act as substrates to generate DSBs (125). Analyses of parasite populations (119) and genetic crosses (74, 77) showed that the subtelomeric regions have a high rate of recombination during the sexual stages. Further studies are needed to ascertain whether these events occur during meiosis or the numerous subsequent mitotic divisions in the mosquito vector.

Copy Number Variation

One of the best-characterized phenotypes of gain-of-function events is the acquisition of drug resistance by copy number variation (CNV). CNVs, either amplifications or deletions, change the number of select genes within a genomic region in order to alter their total expression levels. Original analyses primarily used pulsed-field gel electrophoresis (PFGE) to separate chromosomes, but now CNV detection relies more heavily on quantitative real-time PCR (qRT-PCR) (126), high-density tiling microarrays (127), or whole-genome sequencing (117).

Copy number variation and drug resistance. The classic example of the relationship between CNVs and drug resistance is the increased tolerance to mefloquine conferred by the amplification of a genomic region (amplicon) on chromosome 5 containing the *P. falciparum* multidrug resistance gene, *pfmdr1*, which encodes the P-glycoprotein homolog (Pgh-1). The W2 parasite, initially derived from a Southeast Asian isolate, was pressured *in vitro* with a stepwise increase of mefloquine over 96 weeks, generating the mefloquine-resistant parasite W2-mef (128). Characterization of W2-mef and its derived clones revealed that the key to resistance was the amplification of *pfmdr1*. This results in increased Pgh-1 expression, which is thought to more effectively transport mefloquine into the digestive vacuole, away from its primary site of action (129, 130). Amplifications in other regions in the genome can also lead to increased drug tolerance to a variety of antimalarials. These are listed in Table S1 in the supplemental material.

Additionally, genomic deletions have been described for many parasites. Some prominent examples come from the deletion of unnecessary genes in culture-adapted parasites (e.g., Dd2). Continuous *in vitro* culture is absent of host-derived selective pressure; therefore, functions such as cytoadherence to endothelial cell surface receptors in the microvasculature (131, 132) or gametocytogenesis (133), required for transmission to mosquitoes, can readily be lost.

CNVs are generally believed to be detrimental to cell fitness due to the imbalanced dosage of gene products, thereby perturbing cellular homeostasis. For example, changes in gene dosage have been linked to cancer and neurological disorders, such as autism (134). In *P. falciparum*, the fitness costs to parasite growth

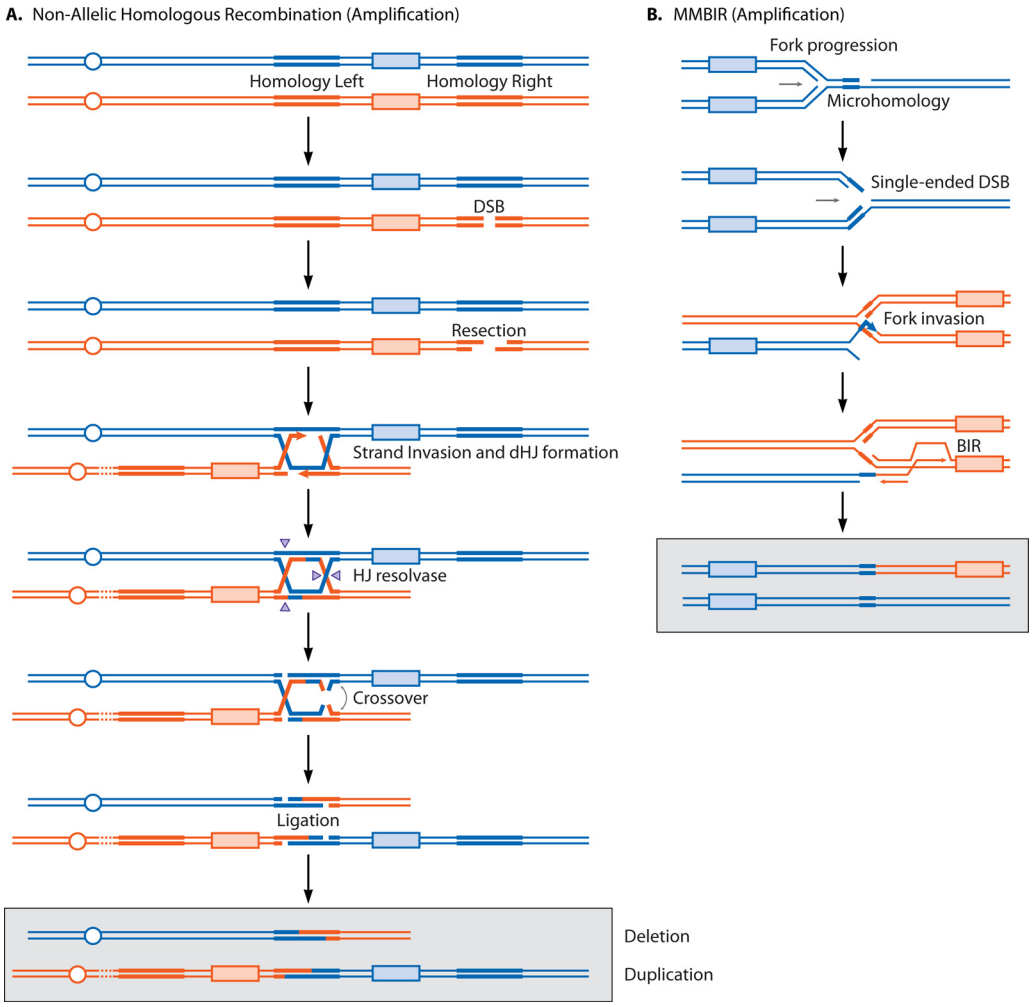

FIG 5 Mechanisms of copy number variation (CNV). (A) Nonallelic homologous recombination (NAHR) can generate genome amplifications or deletions. In NAHR, a DSB is repaired using an incorrect template. Resolution of the dHJ intermediate forms a crossover, which leads to a duplication of a segment (amplicon) on one sister and a deletion on the other. (B) Microhomology-mediated break-induced replication (MMBIR) can generate amplifications or deletions. DNA replication fork progression through a nick will generate a single-ended DSB. Microhomology can then anneal to microhomology on a replicating sister chromatid and undergo BIR to recapitulate the remaining chromosome arm.

have been observed with amplifications of *gch1* (106) and *pfcrt* (our unpublished results). However, in the context of selective or diverse environments, CNVs can prove advantageous in promoting adaptability, as in the case of *in vitro* culture growth and drug resistance. Other CNVs, such as an amplification of adjacent *plasmepsin2* and *hap* (histo-aspartic protease) genes found in a Thai field isolate, have been speculated to increase nutrient acquisition and may be beneficial for the parasite (132). Furthermore, increasing the number of copies of genes that confer drug resistance can allow for one copy to maintain the wild-type sequence and functionality, while the additional copy or copies may mutate to confer drug resistance (126). Therefore, the investigation of parasite CNVs may prove to be increasingly important, particularly in the context of varying geographical drug landscapes.

Possible mechanisms of copy number variation. The mechanism by which CNVs occur has yet to be proven for *Plasmodium*, but a few pathways stand out as likely candidates: NAHR (Fig. 5A) and BIR, particularly microhomology-mediated BIR (MMBIR) (Fig. 5B) (135). Given its presumed absence, NHEJ is not likely to play a role in CNV formation. Additionally, due to the large average amplicon size, it is unlikely that polymerase slippage during DNA replication is a major source of CNVs (135). Therefore, it is presumed that NAHR and/or MMBIR is the primary mediator.

NAHR differs from HR only in that the homology search performed by Rad51 does not use the correct, corresponding sister locus for repair, and in the process amplifies or deletes a given genomic region (117, 135). Given that NAHR requires a template for repair, it is possible that it occurs not only during the mitotic asexual blood stage (126) but also during the mosquito stage, where both homologous chromosomes during meiosis and sister chromatids during sporogony are available (Fig. 1) (136). Evidence of NAHR during the mosquito stage is discernible in the progeny of experimental *P. falciparum* genetic crosses (81).

Amplicons have been found to vary in size from <5 kb to >100 kb. Despite this variation, sequencing of the newly formed junctions (breakpoints) shows a strong preference for monomeric A/T sequences that average about 30 bp (81, 136, 137), which are enriched in untranslated regions. Monomeric tracts of >10 bp that

speckle the genome are estimated to be, on average, about 600 bp apart, or 5% of the genome (136). It may be that one consequence of the genome's A/T richness is to increase the likelihood of copy number variants.

These short, monomeric tracts are possible substrates for MMBIR (Fig. 5B). In this process, a short, monomeric tract revealed by end resection anneals to another microhomology region on another chromosome, independently of Rad51. This tract then serves as a primer for polymerase extension until the telomere, thereby copying that chromosome arm. Recently, the initial mitotic amplification of chromosomal regions around the *pfdhodh* gene in response to the drug DSM1 was speculated to be MMBIR based (126). Amplicons, which varied in size for each clone, ranged from 34 to 95 kb and were arranged head to tail. Breakpoints were short, monomeric tracts. As DSM1 pressure increased, the amplified region containing *pfdhodh* was further amplified. But, importantly, all amplifications were exact copies of the initial amplicon, implicating a faster, homology-based expansion, such as NAHR, as the causal pathway. Therefore, it is possible that MMBIR (Fig. 5B) occurs at a low frequency in the parasite, generating amplicons of various sizes. The close proximity of these amplicons presumably provides substrates more ideal for NAHR than for MMBIR under increasing selective pressure, thereby creating exact copies of the initial amplicon (Fig. 5A).

Together, the evidence collected from a number of studies depicts a parasite that generates copy number variants at a low but significant frequency. Many may be detrimental to growth and fail to establish themselves at the population level. Given the sequence identity of breakpoints and types of amplicons produced, the parasite may employ several of these pathways as tools to increase its genome diversity.

Genome Editing

Genetics in *P. falciparum* is currently becoming increasingly accessible with the nearly simultaneous arrival of new transfection-based technologies, which will undoubtedly deepen our understanding of this parasite and lead to a more thorough definition of parasite determinants of drug resistance, fitness, pathogenesis, and overall cellular organization and developmental biology. The common factor among all the old and new technologies is the requirement for a DSB to initiate HR. It was recognized early in yeast and mammalian systems that the introduction of a single DSB significantly increases the rate of recombination (10, 11). Genetic research in malaria was not too far behind in recognizing the potential of DSBs, though several hurdles have prevented their capitalization, until recently.

Established technologies. Early transfection and mutant parasite generation began around the mid-1990s for both *P. falciparum* and *P. berghei*. Stable transfection of *P. falciparum* originated by introducing an episomal copy of the chloramphenicol acetyltransferase selectable marker (138). This was quickly followed by plasmid integration of a selectable marker, the dihydrofolate reductase-thymidylate synthase (*dhfr-ts*), into the genome, by a method referred to as "single-site crossover" (139, 140) (Fig. 6A). Simultaneously, transfections in *P. berghei* had also produced resistant *dhfr-ts* parasites with both episomal and integrated plasmids (141, 142). Both species were transfected by electroporation, using ring stages for *P. falciparum* and merozoites in the case of *P. berghei*.

Although the number of selectable markers increased (143–145) and alternate transfection methods were developed (146), genetic manipulation techniques and efficiency did not change drastically for *P. falciparum*, aside from the introduction of the "double crossover" technique (Fig. 6B). This technique uses negative in addition to positive selection to remove unwanted recombination events (147, 148). These methods still yield low efficiencies, at best 1 in 10^5 parasites per transfection (149, 150), and require 2 to 3 months of continuous culture, at minimum, to produce desired recombination events at a high enough rate to clone the appropriate parasites. Mechanistically, double crossovers may occur either by SDSA (Fig. 6B) or by integration of the plasmid via single-site crossover followed by crossover-based excision of the negative marker by use of homology within the plasmid.

In contrast to the fate of *P. falciparum* genetic manipulation, methods in *P. berghei* benefitted substantially from the success of linear DNA transfection. Linear DNA (displaying broken DNA ends) in *P. berghei* was readily incorporated into the genome via "ends-in" and "ends-out" methods, which mirror the standard methods utilized in yeast genetics (151, 152). Use of linear DNA and optimization of transfection technology have reduced the time required to generate mutant parasites to less than a week, with average efficiencies nearing 1 in 100 to 1 in 1,000 parasites per transfection (153). These methods were also shown to work in *P. knowlesi* (154).

In *P. falciparum*, transfection of linear DNA by use of nanosomes has shown modest luciferase expression lasting a few days, but no genomic integration was reported (155). To date, linearized DNA for gene editing has not been successful in *P. falciparum*, even when the plasmid is linearized in cells by use of zinc finger nucleases (ZFNs) (our unpublished results). To date, bioinformatic studies have not identified any differences in DNA repair pathways between *P. falciparum* and *P. berghei* that would explain the markedly different efficiencies in linear DNA-based gene replacement.

Engineered endonucleases. The recent development of three powerful gene-editing technologies has spurred enthusiasm for the future of *Plasmodium* genetics, as ZFNs, Tal effector-like nucleases (TALENs), and the CRISPR/Cas system have been shown to be extremely useful in other research and translational settings. For example, TALENs have been designed for all protein-encoding genes in the human genome (156), and ZFN-mediated inactivation of *CCR5* in CD4 T cells can lower the HIV burden in treated patients (157).

ZFNs and TALENs both act as heterodimers where each monomer contains a DNA-binding domain and the nuclease domain of the FokI endonuclease. The DNA-binding domain, also known as the zinc finger protein (ZFP) region, consists of an assembly of three to six C_2H_2 zinc fingers, which each bind, on average, three bases (14). TALENs share the same architecture as ZFNs but differ in that the DNA-binding domain consists of a tandem array of 34-amino-acid repeat modules where each repeat is identical except for two amino acids, which bind a single base (NI binds adenine, HD binds cytosine, NG binds thymine, and NN binds guanine or adenine) (158). The benefits of TALENs include their simple, modular design, which enables high-throughput construction of a large number of variants to recognize different targets. However, difficulties may arise due to the inherent repetitive nature of the DNA-binding domain sequence and the overall size of the nuclease. Fused to both the ZFN and TALEN DNA-binding domains is the FokI nuclease domain, which, upon DNA binding, homodimerizes and creates a DSB,

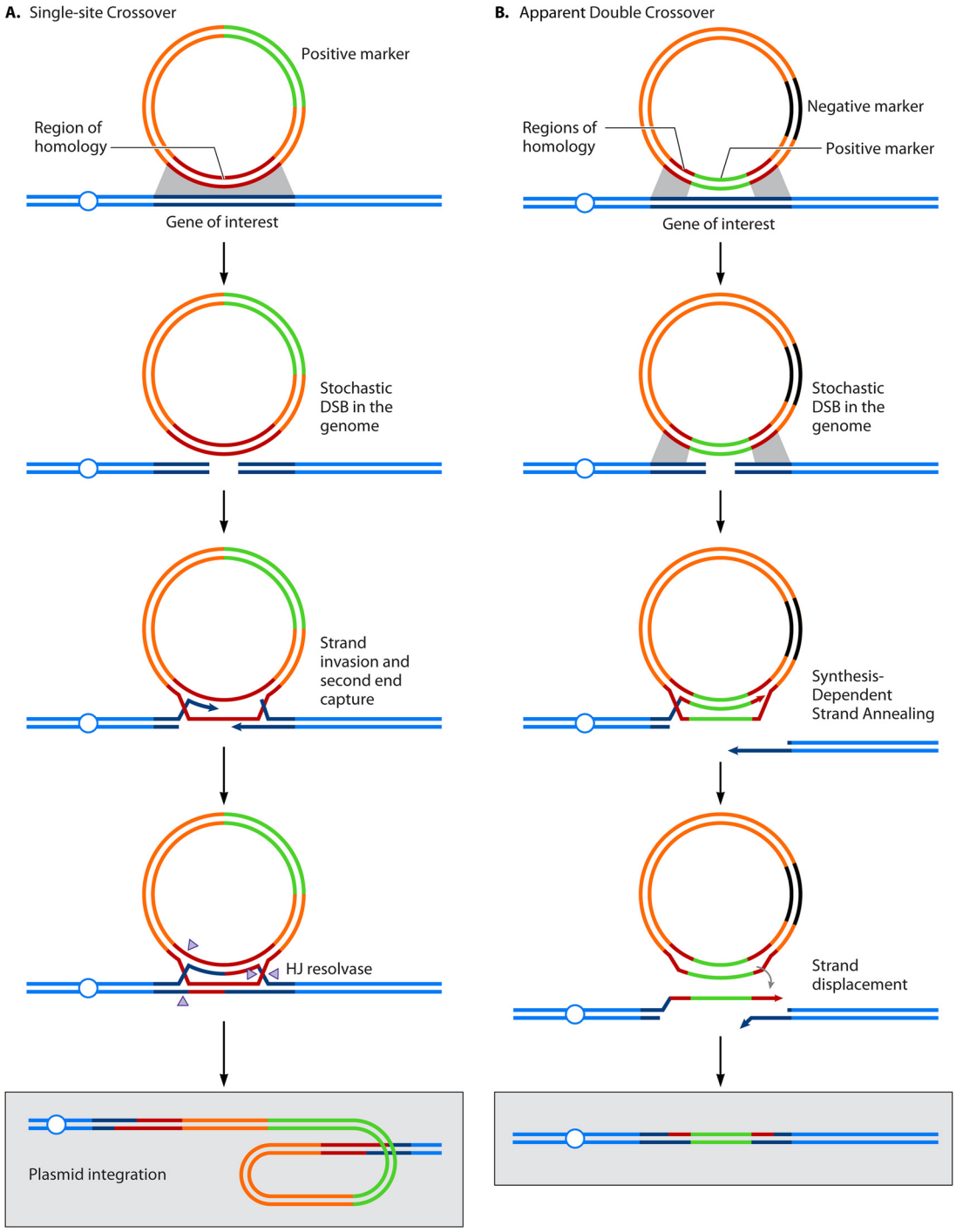

FIG 6 Established technologies for genome editing. (A) Single-site crossover (SSC) was developed to utilize a region homologous to a genomic locus carried on a transfected plasmid with a positive marker (green strands). SSC depends on a stochastic DSB in the genome to provide recombinogenic DNA ends that will use the plasmid region of homology as a template for repair. Formation of a dHJ and its resolution to yield a crossover product will lead to plasmid integration into the genome. (B) An apparent double crossover requires the parasite to incorporate the positive marker placed between two regions homologous to the gene of interest. Recombination (e.g., by SDSA) can copy the positive marker and introduce this into the genome while avoiding the negative marker (dark gray strands). An alternative scenario (not shown) involves two independent crossover events. In this case, the entire plasmid integrates into the genome by SSC and is followed by recombination between the homologous sequences flanking the negative marker, thereby looping it out. Selection for both positive and negative markers will select only for parasites that have incorporated the positive marker.

usually generating 4- or 5-bp 3′ overhangs, depending on the sequence that each monomer binds. Enhancements to the FokI domain have been made such that the nuclease heterodimerizes, lowering its potential off-target specificity (159). The resulting DSB can then be repaired from a plasmid template to transfer the desired sequence change to the genome. Repair in the absence of a homologous template can be used to generate indels through EJ, if such a pathway exists in the organism (14).

The use of ZFNs in the *Plasmodium* field is currently gaining traction. Thus far, published genes targeted in *P. falciparum* include *pfcrt* (13), a genome-integrated enhanced green fluorescent protein gene (*egfp*) (13), and the phosphatidylinositol-4-OH kinase [PI(4)K] gene (57). We and other laboratories have also had success with several other genes (unpublished data). ZFNs have also been recently used to target the *P. vivax dhfr* gene (171). Due to the high A/T content of the *P. falciparum* genome, ZFN design is more difficult than that for other species, but nevertheless, it is feasible. Various factors affect the success of gene editing. ZFN cleavage activity in the parasite generally correlates with activity in yeast proxy assays (159; our unpublished observations). Proximity of SNPs to be incorporated into the genome to the site of ZFN cleavage improves the chances of editing (160). The length of the homologous region on the plasmid positively correlates with the rate of recombination (161, 162). Lengths of homologous regions in *Plasmodium* typically range around 1 kb (13) but can be longer (57), depending on plasmid size constraints.

To date, TALENs have been designed *in silico* to target *Plasmepsin V* and have shown functionality in a yeast reporter cleavage assay (163), but no *Plasmodium* TALEN studies have been published to date.

The first uses of the CRISPR/Cas system in *Plasmodium* research have only recently been published (172, 173). This system modifies a prokaryotic viral defense system to cleave a specific genomic sequence harboring a unique motif, using an RNA-guided Cas9 endonuclease (164–166). Cas9 can bind a fusion RNA sequence where one segment is necessary for secondary structure formation and Cas9 binding and the other is complementary to a given target DNA sequence, which Cas9 will cleave, forming a DSB (164). This system circumvents the relatively more arduous engineering requirements inherent to ZFNs or TALENs. However, studies in other model systems show that the limitations of the CRISPR/Cas system lie in the target genomic site criteria. Target genomic sites must carry a 3-bp NGG protospacer-adjacent motif (PAM) (167) adjacent to a 20-bp genomic recognition sequence.

Recent reports in both *P. falciparum* (172) and *P. yoelii* (173) have successfully shown the ability of the CRISPR/Cas system to introduce SNPs into a gene of interest, tag proteins (e.g., with *gfp*), and knock out coding sequence with and without a marker (e.g., human *dhfr*). The outcomes of each experiment showed a high editing efficiency with no detectable amount of undesirable, off-target events. These initial studies offer a glimpse of the promising future of *Plasmodium* genetics and research. Harnessing DSBs enables researchers to utilize the recombination machinery to generate novel parasites. Using these nucleases, combined with a high-throughput method of design and cloning, may prove to be extremely valuable both for parasitology and for other eukaryotic pathogens, as has already been demonstrated for higher eukaryotes (168–170).

CONCLUSIONS AND PERSPECTIVES

The current understanding of DSBR in *P. falciparum* is in its early stages, as only a few reports directly studying HR have been published. Despite this, meaningful inferences regarding DSBR can be made from studies of antigenic diversification of *var* genes, linkage disequilibrium in genetic crosses, and drug resistance-associated gene amplifications. Whole-genome sequencing and microarray analyses also provide insights into processes such as BIR and CNV. Yet large gaps in our understanding of DSBR still exist. The absence of clear evidence of NHEJ and alternative EJ pathways also remains perplexing. Nevertheless, the recent gain in popularity of genome-editing technologies is putting greater focus on DSBR mechanisms in *P. falciparum*. Understanding the nuances of DSBR will better enable the use of these technologies to gain insights into virulence, pathogenesis, drug resistance, and drug modes of action for one of the most pernicious pathogens encountered throughout human history.

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